RNP transport in cell biology: the long and winding road

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Abstract

Regulation of gene expression is key determinant to cell structure and function. RNA localization, where specific mRNAs are transported to subcellular regions and then translated, is highly conserved in eukaryotes ranging from yeast to extremely specialized and polarized cells such as neurons. Messenger RNA and associated proteins (mRNP) move from the site of transcription in the nucleus to their final destination in the cytoplasm both passively through diffusion and actively via directed transport. Dysfunction of RNA localization, transport and translation machinery can lead to pathology. Single-molecule live-cell imaging techniques have revealed unique features of this journey with unprecedented resolution. In this review, we highlight key recent findings that have been made using these approaches and possible implications for spatial control of gene function.

Introduction

Localization of messenger RNAs (mRNAs) is a conserved mechanism throughout evolution in which gene expression is coupled to its site of function within the cell. This conservation is effected through proteins that have co-evolved to carry these mRNAs to their destinations in a variety of specialized cells and organisms. Although most research has focused on describing mRNA movement, less is known about the unseen actors in the process, the dynamic and changing protein complexes that manage this entire journey from birth to death.

The purpose of this entire process is to regulate the production of proteins in the cell in the place where they function. Many of these proteins are localized post-translationally,

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asymmetric subcellular distribution of protein is efficiently accomplished by on-site activation of translation after the mRNA is transported and localized in a dormant state. This spatial distribution is a key determinant for many aspects of cell structure and function [1,2]. It has been revealed that a large number of transcripts localize to specific subcellular compartments in polarized cells. High-resolution fluorescence *in situ* hybridization (FISH) during early *Drosophila* embryogenesis showed that over 70% of mRNAs studied (20% of total genes) localize to different subcellular compartments, where they usually colocalize with the proteins they encode [3]. Although this spatial distribution of gene expression was originally thought to be a unique feature of highly specialized and polarized cells such as neurons and germ cells, it has been demonstrated that a subset of mRNAs localize to cell protrusions and focal adhesions in migrating fibroblasts [4-8]. High-resolution microscopy has shown that mRNAs encoding inner-membrane proteins are confined at this membrane in *E. coli* [9] [10]*. This indicates that prokaryotes as well as eukaryotes can spatially regulate gene expression modulating the destiny of mRNAs and cell function. Defects in RNA localization machinery have been implicated in disorders ranging from neurodevelopmental and neurodegenerative diseases to cancer [11,12].

In order to understand how RNA moves within the cell, it was imperative to develop high-resolution microscopy technologies directed to follow single molecules of mRNA in living cells from the time they are synthesized in the nucleus until the time they are degraded in the cytoplasm. Intracellular localization of mRNAs was originally observed using *in situ* hybridization in fixed samples [5,13]. It remains the standard tool for examining the distribution of mRNAs in cells, tissues, and even entire organisms at a specific time point. The direct observation of mRNA using the MS2 system in living cells (Box 1) [14-17] [18]** provided the dynamics important for ascertaining mechanism. Recent advances in live cell imaging [19]** have demonstrated that it is possible to follow the mRNA and associated proteins, thereby providing insights into various regulatory steps in the journey.

This Review highlights recent discoveries concerning how mRNPs carry genetic information from the genes to various locations within the cell in order to target protein synthesis to the right place and at right time.

Messenger RNPs control the journey from birth to death

After being born in the nucleus, all molecules of mRNAs start a journey to the site(s) of translation and degradation in the cytoplasm (Figure 1). Transcription is the first step of gene expression regulation. Single-molecule live-cell imaging techniques have revealed that gene promoters stochastically transition between active and inactive transcription states generating bursts of nascent transcripts [20-22]. It has also been shown that these promoter sequences can couple transcription with translation and degradation [23-25] [26]* [27]*. This allows mRNAs to be *"flagged"* for sorting to specific subcellular locations from the moment of their birth in the nucleus. A large number of proteins associate with pre-mRNAs to promote crucial modifications such as the addition of the cap structure to the 5′-end by cap-binding protein, the addition of poly(A) to the 3′-end by polyadenylation proteins, and the deposition of the exon junction complex (EJC) proteins with the removal of introns during splicing [2,28]. The association of these and other RNA-binding proteins (RBPs)

with the transcript during these events, and when the mRNA is exported to the cytoplasm, is essential and directs the mRNA to its next destination [1,29]. For instance, in *Drosophila* oocytes, splicing creates the spliced *oskar* localization element (SOLE) that together with EJC deposition mediate proper mRNA transport to the posterior pole [30-32].

By observing the mRNA tagged with MS2, we can divine how the movement occurs. Surprisingly, most of the movement is by diffusion. Messenger RNPs exhibit Brownian movement and disperse throughout the entire nucleus before they exit, even when the transcription site (TS) is located near the nuclear periphery [33,34]. Once the mature transcript has been released from the TS it moves randomly until it finds nuclear pores in a time frame of a few minutes [35-37]. The nuclear pore complex (NPC) mediates nucleo-cytoplasmic transport through the nuclear envelope. A super-registration approach using fluorescence microscopy revealed that the kinetics of mRNP export in mammalian cells includes three-steps: docking (80 ms), translocation (5-20 ms), and release (80 ms) [37]. Remarkably, mRNPs remain docked at the nuclear basket for a period of time indicating a rate-limiting step possibly related to quality control [37-39]. This would allow crucial protein rearrangements before export. In budding yeast, mRNAs scan the nuclear periphery before being exported, presumably by a receptive pore complex [40]*. This scanning behavior is defective in nuclear basket protein MLP1/2 mutants that shortens the docking time and releases the mRNAs before they can export. It has been shown that not all pores are equally active [37,38], however, pores transporting β-actin mRNA are repeatedly active over time [41]*. Export rate is influenced by mRNPs involved in splicing, alternative polyadenylation and nuclear surveillance as well as the length of the transcript (reviewed in [29,42,43]). For instance it has been shown that unspliced mRNAs are exported very inefficiently as the recruitment of export factors may be faulty. Export directionality is achieved by mRNP remodeling as the mRNA moves through the NPC [40,44]*. The nuclear export factor 1 (NXF1) and NTF2-related protein 1 (NXT1) interact with different adaptor proteins mediating sequential mRNA maturation and export [45]. It has been suggested that in neurodegenerative disorders, where deposition of aggregates of misfolded proteins accumulates in the cytoplasm, nucleo-cytoplasmic RNA export could be affected due to a sequestration and mislocalization of export/import factors [46].

The NPC may not be the only nucleo-cytoplasmic exit pathway. In *Drosophila* neurons, large mRNP complexes appear to leave the nucleus via budding [47,48], a similar mechanism previously shown for nuclear exit of herpes-type virus [49,50].

Upon arrival into the cytoplasm, the mRNP undergoes rearrangements that promote its journey towards its biological destiny. The mRNP composition is remodeled near the nuclear envelope before cytoplasmic release. For instance, Mex67p is removed from the mRNP complex presumably preventing further interaction of the now cytoplasmic mRNA with the pore complex in budding yeast [44]*. The now cytoplasmic mRNP may gain additional factors that control its mobility and further localization (reviewed in [29,43]). In addition to RBPs, noncoding RNAs and microRNAs might be components of these mRNPs [51]. In the cytoplasm, the mRNA movements have a directional component. The mRBPs may directly or indirectly bind to motor proteins (i.e., kinesins, dyneins and myosins) to form high molecular weight mRNP motor complex(es) [29]. In *S. cerevisiae*, *ASH1* mRNA is bound to

She2p, which in turn allows the binding of She3p and Myo4p (myosin) to the complex. Once bound, Myo4p transports *ASH1* mRNA to the daughter cell along actin filaments [52,53]. Single-molecule live-cell imaging and tracking analysis have revealed dynamics of mRNAs as they move through the cytoplasm. The movement of mRNAs can be segmented into stationary, corralled, diffusive, and directed transport [54]. Similar to the nucleus, mRNAs are predominately governed by diffusion movement in the cytoplasm [55]* [18]**. However, they are continually subjected to cycles of diffusion, direct transport and anchoring as shown in neurons by hidden Markov modeling (HMM) [56]. The diffusion coefficient in cytoplasm is faster than in the nucleus because the environment is less restrictive. RNA transport is biased by molecular motors that mediate longer trajectories through direct association with the cytoskeleton [57] and hence bring mRNAs into subcellular regions where they are more likely to anchor awaiting the right time to be translated. The ability of some mRNAs to become localized and translated at specific regions has profound implications for cell structure and function. The best example of this is the neuron. Messenger RNPs have to travel from the cell body to synapses that are far away, sometimes meters, to be locally activated to synthesize proteins at a precise moment. This mechanism is the basis of learning and memory [58]. For instance, β-actin mRNA faces this logistic problem in hippocampal neurons by continuously assembling and disassembling large mRNA-protein complexes while traveling towards the base of activated dendritic spines [18,59,60]**. It has been shown that diffusion of β-actin mRNA in neurons is slower than in fibroblasts and 10% of mRNAs are actively transported with a mean speed of 1.3 µm/s, equivalent to the assembly with a microtubule motor complex [18]**. Newly synthesized β-actin protein occurs in activated spines [60]**. This local production of actin protein allows enlargement of the cell structures involved in synaptic growth and arborization in neurons [61-64] [60]**. It has been shown that this local translation regulation of β-actin mRNA involves translational de-repression by Src kinase-mediated phosphorylation of the zipcode binding protein 1 (ZBP1) [65]. Arc mRNA (which encodes activity-regulated cytoskeleton-associated protein) is also targeted to the base of individual dendritic spines where synapse-specific translation may occur [66]. Similarly, localization of *oskar* mRNA to the posterior pole is essential for germ cell formation during *Drosophila* oogenesis [67]. *oskar* mRNA diffuses randomly, with only 13% being actively transported [68]. The RNA transport towards the posterior pole may be favored by a subtle bias in microtubule orientation. Translation of *oskar* mRNA is repressed by Bruno during transport [69,70] and it is only active when it reaches the posterior pole [71-73]**.

Innovative improvements in reagent design using rapid live fluorescence microscopy, made it possible to determine when and where single molecules of mRNAs translate [73-78]** (Figure 2a,b). It is now possible, using fluorescence fluctuation spectroscopy (FFS, Figure 2c), to ascertain that the association of a specific RBP (ZBP1) with a specific mRNA (β-actin) in living cells is anti-correlated with the assembly of ribosomes, hence validating its role in translational repression and its dissociation at the periphery where actin protein translation then occurs [19]**. It has been thought that mRNA translation was silenced during transport in neurons but recent tracking indicated that the mRNA can be in the act of translating while moving: 20% of mRNAs are both actively translated and transported along dendrites at 2 µm/s [74].

Most mRNA-polyribosome complexes (polysomes) are diffusive in the cytoplasm [19,74,77]**. It has also been shown that the association of the transcript with the translation machinery slightly affects their mobility [74]*. This provides another approach to determine when and where mRNPs are translated. Tracking of thousands of mRNA-ribosome trajectories per cell showed that ribosome load slightly slowed down highly translating mRNAs in fibroblasts [55]* (Figure 2d). For endoplasmic reticulum (ER)-associated polysomes the mobility is slower than for free cytoplasmic counterparts as the nascent peptide restricts the movement to membranes [74,77]**.

A combination of cell biology, biochemistry and genome-wide approaches have showed that each mRNA is bound by multiple RBPs and that individual RBPs can be associated with hundreds (and possibly thousands) of target mRNAs. Elucidation of the molecular mechanism of RNA transport requires the identification and characterization of these RBPs (and motors) as well as the *cis*-acting regulatory elements present in the mRNA. Many RBPs have been identified and deduced from sequence homology; however, only a handful of them had been validated *in vivo*. A step towards the understanding of protein binding to mRNA is the recent global initiative to validate RBPs (https://www.encodeproject.org/ [79]*). The RBPs bind to localization elements (LE) located mainly in the 3′-UTR. They can be either primary nucleotide sequences or secondary structures. Less frequently these regulatory elements can be also found in the 5′-UTR, coding sequence or introns (reviewed in [2,80]). For mRNAs coding for membrane associated proteins, the nascent peptide can target the entire translating mRNP to its final destination: mitochondria or the ER [81,82] [74]* [77]*. LEs show modularity, redundancy and diversity. The combinatorial code of LEs and their binding factors specifies the destiny of the mRNP and its effect on cell physiology. Messenger RNAs that code for functionally related proteins might be transported, localized and translated together. For instance, proteins required for spindle formation and hence for meiosis progression in *Xenopus* oocytes are synthesized synchronously by mRNAs localized on spindle microtubules [83]. Similarly, localization of mRNAs encoding for proteins involved in focal adhesions controls cell adherence and motility of fibroblasts [4,84] [55]*. Local translation of β2B-tubulin mRNA near to microtubule tips in axons and growth cones has been proposed to promote neuron migration [85]*. These events described above all have their respective RBPs controlling their localization and translation. Some of these proteins have been identified. The cytoplasmic polyadenylation binding element protein (CPEB) is involved in the microtubule-localized translation activation of mRNAs encoding for spindle proteins. The ZBP1 has been shown to be essential for motility related mRNAs in fibroblasts and the localization of mRNAs within dendritic compartments of neurons. The Adenomatous polyposis coli (APC) mediates translation at microtubule ends but is an unconventional RBP in that it doesn't contain known RNA binding motifs.

Regulation of mRNAs in time and space also requires degradation. mRNA decay includes mechanisms of quality control that eliminate the synthesis of possibly toxic peptides and that shorten mRNA half-life, changing the abundance of a functional protein [86]. For instance, single-molecule imaging revealed that non-sense mediated decay (NMD) takes place immediately after nuclear export [87,88]. Similar to translation, mRNA decay occurs in the cytoplasm. It has been shown that degradation as well as translation can be determined in the

nucleus for stress-response and cell cycle-regulated genes in mammalian cells and yeast [23-25] [26]* [27]*. This suggests that specific mRNPs integrate and regulate events between the cytoplasm and the nucleus. However, the mechanism of this communication remains to be elucidated.

Conclusions

The movements of RNPs from one compartment of the cell progressively to the next and the regulatory events surrounding each step illustrate an elegant integration of spatiotemporal events within a single cell. Over the last few years, it has become clear that this mechanism defies thermodynamics by orchestrating a highly complex pattern of subcellular protein distribution, effected by specific proteins that bind to and regulate the mRNAs that code for these cellular distributions. Single-molecule single-cell imaging has revealed mechanistic features of this RNA localization, transport and translation with unprecedented spatial and time resolution. Despite recent findings, it is still not possible to follow an individual mRNA and the proteins with which it associates in a living cell *all the way* from its site of transcription in the nucleus to the site of translation with precise kinetics for all the steps. Hence, although mRNA behavior is well described in living cells much of the mechanism is still unknown and it includes coordinated regulation by RBPs in time and space, including non-coding RNAs. Future efforts on the characterization and visualization of individual mRNPs for these molecules during their journey will eventually explain how this is related with cellular function and disease.

Acknowledgments

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Highlights

- RNA localization is highly conserved throughout evolution.

- mRNPs regulate every step of the journey from birth to death.

- mRNPs movement is a combination of diffusion and directed transport.

- RNA localization is coupled to translational control for local protein synthesis.

- mRNAs can be simultaneously transported and actively translated in neurons.

Box 1

The MS2 System

The MS2 system has been successfully used to visualize mRNAs in living cells and whole organisms due to its simplicity and high sensitivity [14,17,18,54,89,90]. This binary system relies on tagging RNA with fluorescent proteins (FPs) in vivo. It is based on the high-affinity association between a specific and unique RNA stem-loop structure derived from the MS2 bacteriophage genome regulatory element and the bacteriophage MS2 coat protein (MCP) fused to a FP [14,91]. The amplification of the fluorescent signal is achieved by the generation of multimerized MS2 stem-loops or binding sites (MBS) inserted in the gene of interest that can be recognized by an increase number of molecules of MCP-FP, hence, allowing the visualization of single-molecules in living cells due to an increase in the signal-to-noise ratio [92]. Similar to the MS2 system, the interaction between the Pseudomonas aeruginosa PP7 bacteriophage coat protein (PCP) and their cognate RNA stem-loop has been characterized [91,93] and has been also used for visualization of single-molecule mRNAs in living cells [73,76,77,94]. MS2- and PP7-engenieried transcripts can be used simultaneously for two-color imaging of different mRNA species allowing the study of trafficking dynamics for different mRNA species within the same cell, thus expanding what can be studied in living cells.
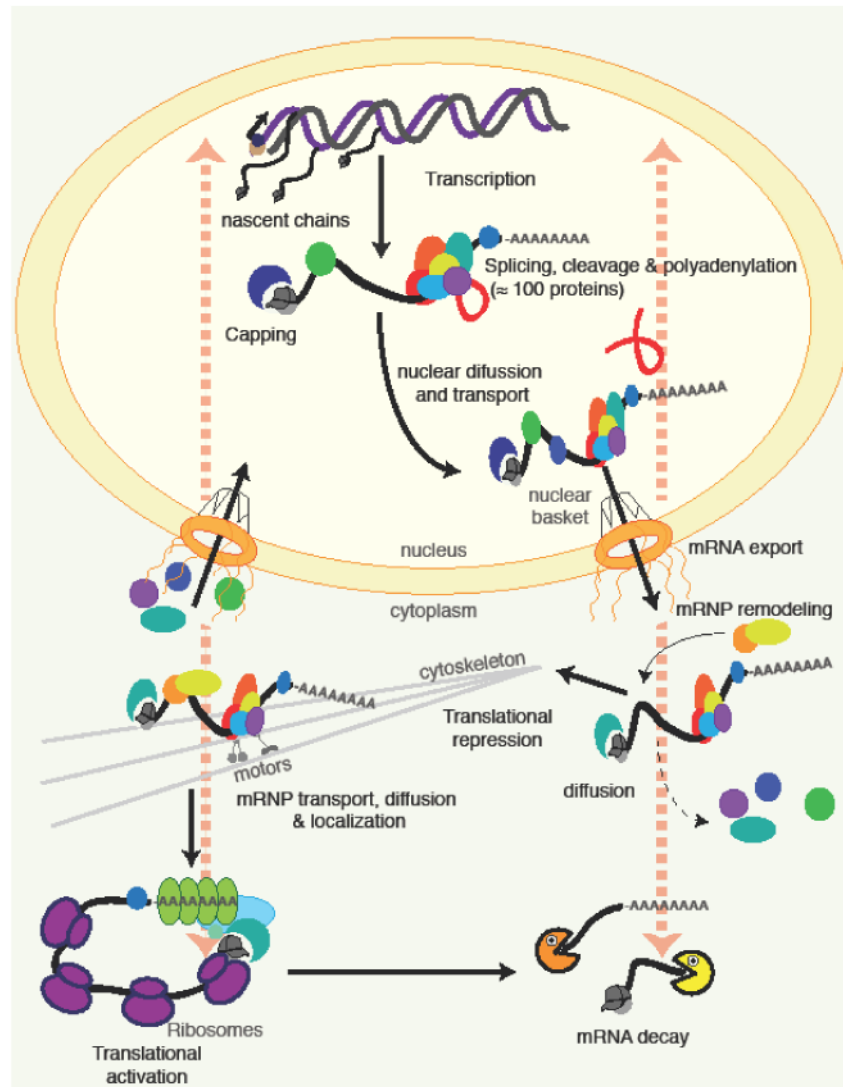
Figure 1. Messenger RNPs control the journey from birth to death

Dynamic association with RNA-binding proteins regulates the journey of an mRNA from transcription in the nucleus to translation and degradation in the cytoplasm. In the nucleus, pre-mRNAs are capped, spliced, cleaved and polyadenylated co-transcriptionally but then diffuse to nuclear pore complexes. Quality control mechanisms ensure that only properly processed mRNPs are remodeled and exported to the cytoplasm, where they undergo a series of diffusional and transported steps. Continuous mRNA remodeling allows proper RNA transport and localization to the subcellular sites of translation and degradation. Dotted orange arrows represent communication between the cytoplasm and the nucleus.
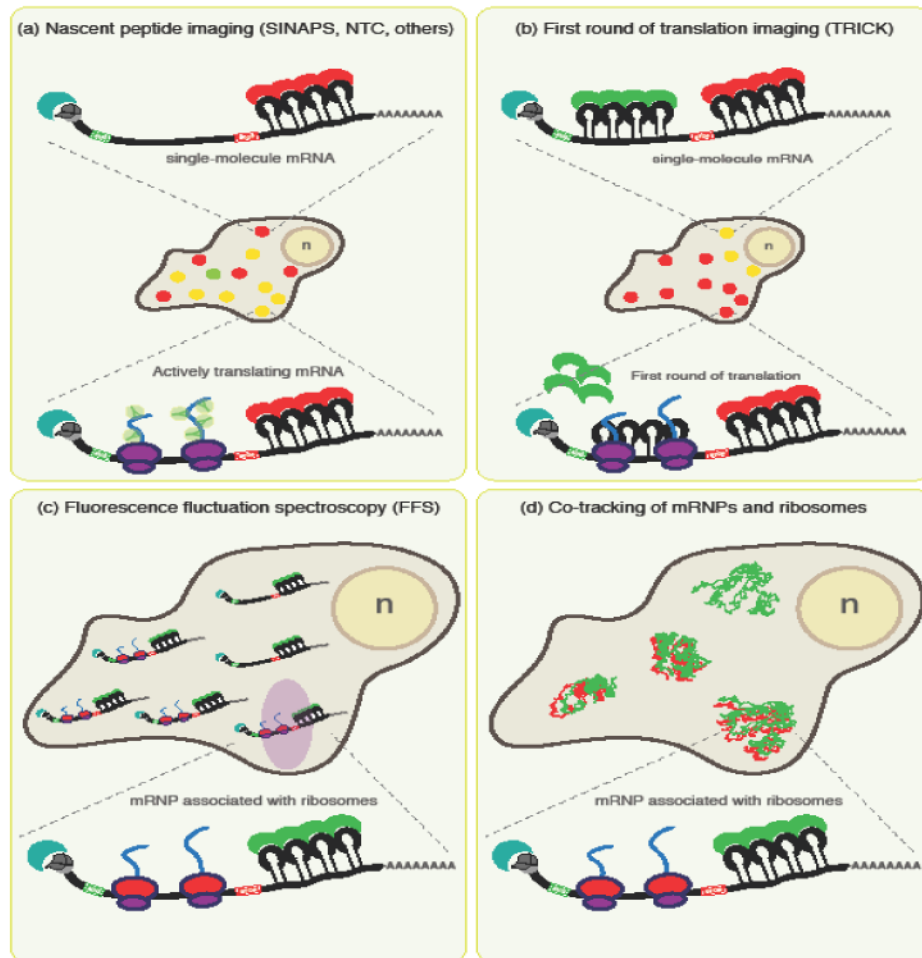
Figure 2. Single-molecule live-cell imaging techniques that monitor mRNP dynamic translation
(a) Nascent peptide imaging techniques (SINAPS, NTC and others) [74-78] visualize
translation in real time. RNA reporter construct includes a labeling tag for the nascent
peptide (SunTag or FLAG) in the coding sequence and MS2 or PP7 stem-loops in the 3′-
UTR for labeling the mRNA. Co-expression of fluorescent antibodies that interact with
SunTag and FLAG (green) allows the visualization of nascent peptide chains (blue).
Fluorescent proteins (FPs) fused with the MCP or PCP (red) bind to the 3′-UTR and enables
mRNA tracking. Actively translating mRNAs are visualized as yellow spots. (b) Translating
RNA imaging by the coat protein knock-off (TRICK) method [73] discriminates between
mRNAs that have undergone the first round of translation (red spots) and mRNAs that have
never been translated (yellow spots) by tagging both the coding sequence and 3′-UTR with
RNA stem-loops that bind distinct FPs (green and red). The FP bound to the tags in the
coding region is knocked-off by the translating ribosome. (c) Fluorescence fluctuation
spectroscopy (FFS) [19] allows spatiotemporal quantification of the association between
fluorescent labeled ribosomes (red) and MS2-tagged mRNAs (green) as they pass through a
femtoliter volume illuminated by a two-photon spot. (d) Co-tracking of fluorescent labeled

ribosomes (red) and mRNAs using the MS2 system (green) shows translation "hot-spots" and dynamics of polysomes in living cells [55].